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Rab Effector EHBP1L1 Associates with the Tetratricopeptide Repeat Domain of Kinesin Light Chain 1

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Kinesin-1 is microtubule-dependent plus-end direct molecular motor protein essential for intracellular transport. It is a member of the kinesin superfamily proteins (KIFs) which transport cargo, including organelles, vesicles, neurotransmitter receptors, cell-signaling molecules, and protein complexes through interaction between its light chain subunit and the cargo. Kinesin light chain 1 (KLC1) is a non-motor subunit that associates with the kinesin heavy chain (KHC). Although KLC1 interacts with many different adaptor proteins and scaffolding proteins, its binding proteins have not yet been fully identified. We used the yeast two-hybrid assay to identify proteins that interact with the tetratricopeptide repeat (TPR) domain of KLC1, and found an interaction between KLC1 and EH domain-binding protein 1 like 1 (EHBP1L1). EHBP1L1 bound to the region containing all six TPR repeats of KLC1 and did not interact with KIF5B (a motor protein of kinesin 1) or KIF3A (a motor protein of kinesin 2) in the yeast two-hybrid assay. The carboxyl-terminus of the coiled-coil domain of EHBP1L1 is essential for interaction with KLC1. However, another EHBP1L1 isoform, EHBP1, did not interact with KLC1 in the yeast two-hybrid assay. KLC1 interacted with GST-EHBP1L1 and its coiled-coil domain but not with GST only. When co-expressed in HEK-293T cells, EHBP1L1 co-localized with KLC1 and co-immunoprecipitated with KLC1 and KIF5B but not KIF3A. These results suggest that kinesin 1 motor protein may transport EHBP1L1-associated cargo in cells.

Key words : Adaptor protein, EHBP1L1, EH domain, kinesin 1, KLC1

Introduction

The intracellular transport is mediated by microtubule-dependent motor proteins such as dynein and kinesin. These microtubule-dependent motor proteins act in several processes in the cell, including cell movement, and membrane dynamics [4, 10]. Kinesin 1 is the first identified microtubule-dependent motor proteins involved in the transport of various cargoes, including membrane vesicles, organelles, proteins complexes, and mRNAs, along microtubule [5]. It is a member of kinesin superfamily proteins (KIFs) for anterograde transport [17]. Kinesin 1 is a heterotetramer composed of two kinesin heavy chains (KHCs, also called KIF5s) and two kinesin light chains (KLCs) [4, 17]. KHC/KIF5 contain three domains: an amino (N)-terminal microtubule-binding motor domain with ATPase activity, a central coiled-coil domain, and a carboxyl (C)-terminal domain that interact with cargo and regulates motor activity [4]. KLCs are non-motor proteins that associate with KHCs. KLCs consist of a N-terminal coiled-coil domain that binds to the KHCs stalk region, a central tetratripeptide repeat (TPR) domains, and a variable C-terminal region [3]. The TPR domain consists of 34 amino acids which assemble into a characteristic helix-turn-helix structure for each TPR repeat [1, 7]. The basic function of TPR domain is well known as a mediator

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domain of protein-protein interaction [1, 3, 20]. The TPR domain of KLC1 consist of six TPR domain (TPR1-6) with a non-TPR domain of 40 amino acids inserted between TPR5 and TPR6 domain [7, 20]. The TPR domains of KLC1 have been suggested to be involved in cargo recruitment of kinesin 1 [3, 20]. The first protein identified to bind to the TPR domain of KLC1 was the c-Jun NH_2-terminal kinase (JNK)-interacting protein 1 (JIP1, also known as islet brain (IB) and JSAP1) [4, 12, 15]. JIP1 is adaptor protein connecting the kinesin 1 to cargo receptors such as apolipoprotein E receptor 2 (ApoER2) and β-amyloid precursor protein (APP) [4, 16, 17].

Kinesin 1 is able to transport various different types of cargos such as mitochondria, neurotransmitter receptor containing vesicle, virus particle, and mRNA granules, along the microtubule [17]. In some cases, these cargoes bind to soluble adaptor proteins/scaffolding proteins that mediate the attachment to kinesin 1 [4, 17].

Understanding how kinesin 1 is able to bind to various cargos and how protein complex assembles on scaffolds at their final destination remain to be unsolved question. In this study, we screened for proteins that interact with the TPR domains of KLC1 and found the interaction with the EH domain-binding protein 1-like 1 (EHBP1L1), Rab8/10 effectors that associates with Bin1 to generate membrane curvature to excise the vesicle at the endocyctic recycling compartment and accumulate on Rab8-positive enlarged lysosomes [2, 11]. The KLC1 and EHBP1L1 interaction suggests that EHBP1L1-associeted Rab8-positive enlarged lysosomes may be transported by kinesin 1, and play a role as a scaffold for between kinesin 1 and Rab8-positive enlarged lysosomes.

Materials and Methods

Plasmid constructs

The mouse KLC1 cDNA fragment corresponding to the six TPR domains-containing region (amino acids 80-End) was cloned into pLexA (Clontech, Palo Alto, CA, USA) [6]. The resulting recombinant plasmid, pLexA-6xTPR-KLC1, was used as bait plasmid this yeast two-hybrid screening. The full-length cDNAs and a series of deletion mutants of mouse EHBP1L1 were subcloned from pcDNA-EHBP1L1 (provided by Professor A, Harada; Osaka University Graduate School of Medicine, Osaka, Japan) [11] into the *EcoR*I and *Xho*I restriction sites of the pLexA or pB42AD (Clontech).

Screening of 6xTPR-KLC1-binding proteins by yeast two-hybrid screening

The Matchmaker LexA two-hybrid system was used for screening according to the manufacturer's manual (Clontech). In brief, pLexA-6xTPR-KLC1 was transformed into yeast strain EGY48 carrying the p8op-lacZ gene. The transformed EGY48 yeast cells containing pLexA-6xTPR-KLC1 were transformed with the mouse brain cDNA library [6, 10] and grown on synthetic dextrose (SD) plates supplemented with glucose but with no histidine, tryptophan, or uracil (SD/-His/-Trp/-Ura). The selection of positive clones was performed on an SD/-His/-Trp/-Ura/-Leu plate containing galactose, raffinose, X-gal, and BU salts. Plasmids from positive clones were analyzed by restriction digestion. Unique inserts were sequenced and protein sequence analysis was performed with the BLAST algorithm at the National Center for Biotechnology Information (NCBI). Sequence-verified clones were tested again for interaction with the bait in yeast by the retransformation.

β-Galactosidase activity in liquid cultures of yeast

The β-galactosidase activity of yeast was assayed as described previously [6]. In brief, mid-log phase yeast cells were collected and permeabilized with 0.1% sodium dodecyl sulphate (SDS) and chloroform. An excess amount of o-nitrophenyl-β-D-galactoside (ONPG) was added to yeast lysate, the mixture was incubated at 30℃, and then the reaction was stopped by increasing pH to 11 by the addition of 1 M Na_2CO_3. The formation of the reaction product, o-nitrophenol, was determined by measuring absorbance at 420 nm on a spectrophotometer and normalizing for the reaction time and the cell density. The units of enzyme activity were calculated by the following equation: units=1,000 × [(OD_{420} - 1.75 × OD_{550})]/ (reaction time x culture volume × OD_{600}). All experiments were independently performed at least three times [6].

Glutathione S-transferase (GST) pull-down assays

cDNA encoding the full-length EHBP1L1 was cloned into pET41a. The recombinant GST- EHBP1L1 and GST- EHBP1L1-coiled-coil domain fusion protein was expressed in *E. coli* strain BL21 GOLD (Stratagene, La Jolla, CA, USA) following induction with 0.5 mM isopropyl thio-β-D-galactopyranoside (IPTG) for 3 hr. The fusion proteins were purified by attachment to glutathione-agarose beads (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's protocol.

The mouse brain S2 fraction was incubated overnight at 4℃ with the GST fusion protein-coupled glutathione beads. The beads were pelleted by centrifugation, washed three times with the extraction buffer [1% Triton X-100 in phosphate-buffered saline (PBS) containing 10 μg/ml each of aprotinin, leupeptin, and pepstatin and 1 μM phenylmethanesulfonyl fluoride], and once with PBS. The bound proteins were eluted from the glutathione beads with 100 μl 1X Laemmli loading buffer. The pulled-down proteins were analyzed by immunoblotting with anti-KLC1 antibody (1:800, cat. no. ab187179; Abcam, Cambridge, MA, USA). The animal study was approved by the institutional review board (IRB), and the approval number was 17-12 of Inje University animal center.

Cell culture and transfection

Human embryonic kidney (HEK)-293T [American Type Culture Collection (ATCC) CRL-3216] cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, L-glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin at 37℃ in a humidified 5% CO_2 incubator. Transient transfections were performed using the $CaPO_4$ precipitation method [6].

Immunocytochemistry

HEK-293T cells grown on poly-D-lysine-coated coverslips were transfected with enhanced green fluorescent protein (EGFP)-EHBP1L1 and KLC1 constructs. Twenty-four hours after transfection, cells were washed with PBS, fixed with 4% paraformaldehyde in PBS for 5 min, and permeabilized with 0.2% Triton X-100 (Sigma-Aldrich) in PBS for 10 min. After blocking with 5% normal goat serum in PBS for 30 min, cells were incubated overnight at 4℃ with anti-KLC1 antibody (1:500, cat. no. ab187179; Abcam) in PBS containing 1% bovine serum albumin (BSA) and 0.05% Tween-20 (Sigma-Aldrich). After washing three times with PBS, cells were incubated for 40 min with Dylight 594-conjugated goat anti-rabbit IgG antibody (1:800, cat. no. 111-516-046; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA). After washing three times with PBS, the cells were mounted with Fluoromount (DAKO, Santa Clara, CA, USA). Fluorescence images were acquired on a Zeiss LSM510 META confocal laser-scanning microscope (Carl Zeiss Inc., Zena, Germany).

Co-immunoprecipitation and immunoblot analysis

Twenty-four hours after transfection with the myc-KLC1 and FLAG- EHBP1L1 constructs, the HEK-293T cells were rinsed with ice-cold PBS twice and lysed with ice-cold lysis buffer [PBS containing 0.5% NP-40 and 1X protease inhibitor cocktail set V (EMD Millipore, Billerica, MA, USA)] by gentle rotation for 30 min. Lysates were centrifuged at 16,000 × g for 10 min at 4℃. The supernatant was incubated with anti-FLAG M2 agarose beads (Sigma-Aldrich) for 2 hr at 4℃ with constant shaking. The beads were collected by centrifugation at 2,000× g for 30 sec and washed five times with ice-cold PBS containing 0.5% NP-40. The washed beads were resuspended with 2X Laemmli loading buffer and the proteins were eluted and denatured by boiling for 2 min. The proteins were processed for 10% SDS-PAGE and immunoblot analysis with antibodies against KLC1 (Abcam), KIF3A [9, 15], KIF5B [10], and FLAG (1:2,000, cat. no. F7425; Sigma-Aldrich).

Results

Identification of KLC1-interacting proteins by yeast two-hybrid screening

To identify the new cargos of kinesin 1, we screened a mouse brain cDNA library through the yeast two-hybrid assays using the six TPR domains-containing region (amino acids 80-542) of KLC1 as bait (Fig. 1B). From $8x10^6$ colonies screened, we obtained 3 positive clones. The positive clones turned out to possess EHBP1L1 cDNA fragments (Fig. 1A). Both positive clones overlapped at the open reading frame (ORF) of EHBP1L1 and possessed cDNA fragments corresponding to the C-terminal coiled-coil domain of EHBP1L1 (Fig. 1A). KLC1 is composed of an N-terminal domain that binds to the stalk region of KHCs, a central six TPR domain that bind the cargos of kinesin 1, and a variable C-terminal region [1, 3, 7].

To narrow the search for the minimal binding domains of KLC1 that is required for the interaction with EHBP1L1, we constructed several different deletion mutants of KLC1. Yeast two-hybrid assays showed that the binding with EHBP1L1 was dependent on the six TPR domains of KLC1 (Fig. 1B). EHBP1L1 was originally discovered as the EH domain binding protein 1-like 1 [9]. It is a multi-domain protein comprised of the C2 domain, the calponin homology (CH) domain, and the coiled-coil domain [11]. To identify the domain of EHBP1L1 required for the interaction with the six TPR domains of KLC1, a series of deletion mutants of EHBP1L1 was constructed and analyzed their interactions

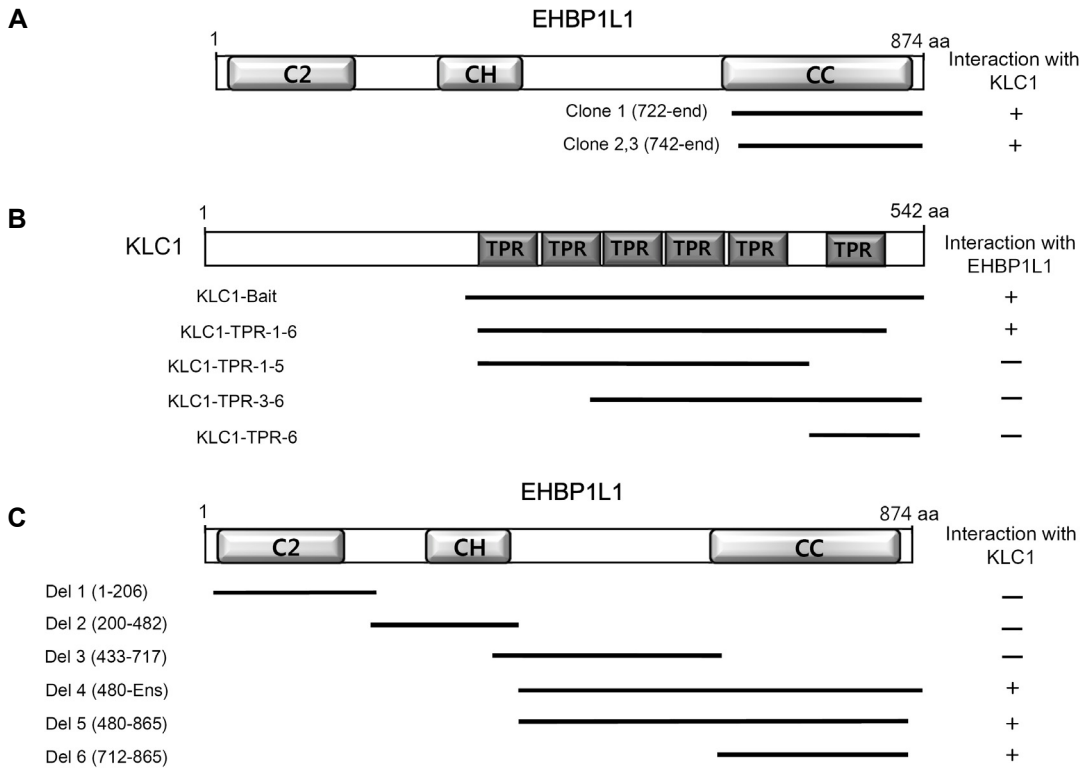

Fig. 1. Identification of the proteins interacting with KLC1 by yeast two-hybrid screening. (A) Schematic diagram of EHBP1L1. The gray boxes indicate the C2 domain, the calponin homology (CH) domain, and coiled-coil (CC) domain of EHBP1L1. Three positive clones isolated from the yeast two-hybrid screening possess the cDNA for EHBP1L1. (B) EHBP1L1 binding region in KLC1. KLC1 has six TPR motifs, indicated in gray. The truncated forms of KLC1 were assessed in the yeast two-hybrid assay for interaction with EHBP1L1. (C) KLC1 binding region in EHBP1L1. Different truncations of EHBP1L1 were tested in the yeast two-hybrid assay for interaction with KLC1. +, interaction; -, no interaction; KLC, kinesin light chain; EHBP1L1, EH domain-binding protein 1-like 1; CH, calponin homology; CC, coiled-coil; TPR, tetratricopeptide repeat; aa, amino acids.

with KLC1 using the yeast two-hybrid assay. Only the coiled-coil domain of EHBP1L1 interacted with KLC1 in the yeast two-hybrid assay (Fig. 1C). This experiment demonstrated that the minimal binding domain was located in the C-terminal coiled-coil domain of EHBP1L1.

To clarify whether EHBP1L1 interacts with only KLC1 or with other KHCs, the C-terminal tails of KIF5B (a KHC of kinesin 1), and the C-terminal tails of KIF3A (a KHC of kinesin 2) were tested for binding with EHBP1L1. As shown in Fig. 2A, EHBP1L1 did not interact with the C-terminal tails of KIF5B, and the C-terminal tails of KIF3A. Next, we investigated whether KLC1 interacts with the other EHBP1, and EHBP1L1. As shown in Fig. 2B, KLC1 did not interacted with EHBP1 in yeast two-hybrid assay. To quantify the binding affinity of KLC1 to EHBP1L1, the EHBP1L1 full length, or EHBP1L1-coiled-coil domain plasmid and the KLC1, KIF5B, or KIF3A expression plasmids were transformed to yeast and the β-galactosidase activity was measured in liquid cultures. The interaction of EHBP1L1 with KLC1 yielded approximately 462 units of β-galactosidase activity (Fig. 2C).

To further confirm the KLC1 and EHBP1L1 interaction at the protein level, the interaction between KLC1 and full length EHBP1L1 or EHBP1L1-coiled-coil domain was assayed suing a GST pull-down experiments. Recombinant GST- EHBP1L1 or GST- EHBP1L1-coiled-coil domain fusion proteins were expressed in *E. coli*. The purified GST fusion proteins are allowed to interact with mouse brain lysates. Immunoblotting analyses revealed that KLC1 interacted with GST-EHBP1L1, and GST-EHBP1L1-coiled-coil domain but not with GST (Fig. 2D). This result indicates that EHBP1L1 associates with KLC1 at protein level.

EHBP1L1 is associated with kinesin 1 in cells

To further confirm the EHBP1L1 and KLC1 interaction in mammalian cells, co-immunoprecipitation from HEK-293T cells that were transfected with FLAG-EHBP1L1 and

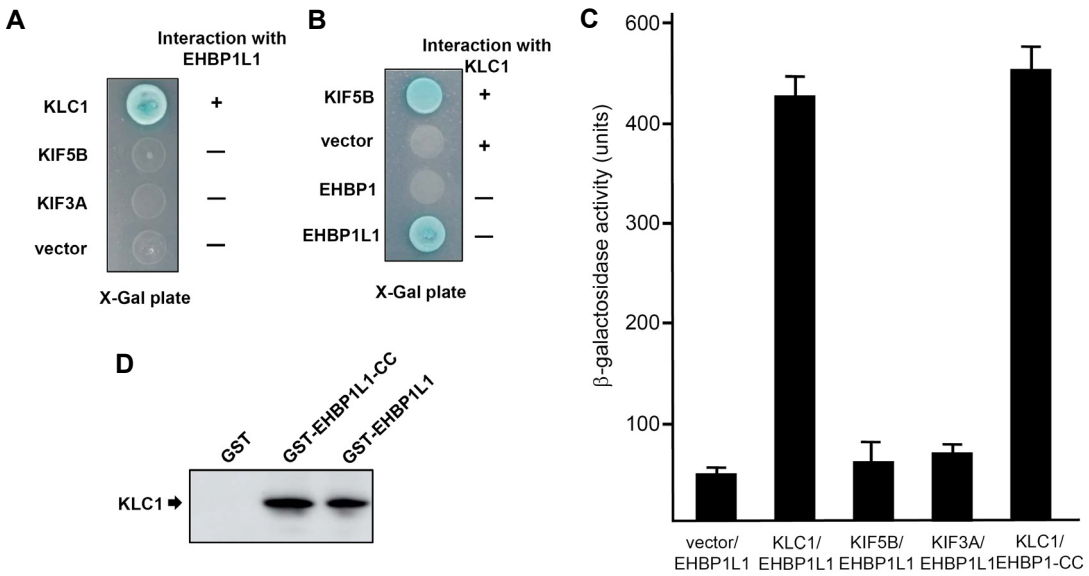

Fig. 2. Interaction of KLC1 or KIFs with EHBP1L1. (A) The tail regions of each KIF and the full length KLC1 were tested for the interaction with EHBP1L1 in the yeast two-hybrid system. EHBP1L1 specifically interacted with KLC1, but not with KIF3A and KIF5B. (B) The tail regions of KIF5B and the full length EHBP1 and EHBP1L1 were tested for the interaction with KLC1 in the yeast two-hybrid system. KLC1 specifically interacted with EHBP1L1, but not with EHBP1. KIF5B served as a positive control for interaction. (C) The strength of interactions between EHBP1L1 and KIFs, or KLC1 was examined quantitatively using β-galactosidase activity in the yeast two-hybrid reporter assay. Values are presented as the mean ± standard deviation. (D) Direct binding of KLC1 to EHBP1L1 in a GST pull-down assay using purified GST-fused EHBP1L1 and GST-fused EHBP1L1-CC. +, interaction; -, no interaction; KLC, kinesin light chain; KIF, kinesin superfamily proteins; EHBP1L1, EH domain-binding protein 1-like 1; CC, coiled-coil; GST, glutathione S-transferase.

myc-KLC1 was performed. Anti-FLAG antibody precipitated KLC1 and endogenous KIF5B; however, KIF3A did not (Fig. 3A). Conversely, anti-myc antibody precipitated EHBP1L1 and endogenous KIF5B, but not KIF3A (Fig. 3B). These results indicate that EHBP1L1 interacts with KLC1 bound to KIF5. In order to address whether KLC1 and EHBP1L1 co-localize in cells, KLC1 was co-expressed with EGFP-EHBP1L1 in HEK-293T cells. KLC1 and EHBP1L1 were identified to co-localize at the same region in cells (Fig. 3C). Taken together, these results indicate that EHBP1L1 is a novel binding partner of kinesin 1 through the binding with KLC1 subunit.

Discussion

In this study, we show that KLC1 interacts with EHBP1L1, as a binding protein of kinesin 1. Using the six TPR domains-containing region of KLC1 as bait, we identified EHBP1L1 in a yeast two-hybrid assay of a mouse brain cDNA library. The C-terminal coiled-coil domain of EHBP1L1 interacts with the six TPR domains of KLC1. Furthermore, when KLC1 and EHBP1L1 were expressed in mammalian cells, they co-immunoprecipitated and co-localized in cells. Taking all of these results together, we hereby propose that KLC1 and EHBP1L1 interaction may have role in formation of a scaffold for between kinesin 1 and Rab8-positive enlarged lysosomes.

EHBP1L1 plays a role as a module that permits the assembly of multi-protein complexes and involved in the regulation of stress-induced lysosomal enlargement and secretion [2, 11]. Previous biochemical studies have shown that the coiled-coil domain of EHBP1L1 binds many Rab-binding proteins, such as MICAL-1, MICAL-L1, MICAL-L2, and EHBP1 [9, 13, 19]. The C2 domain of EHBP1L1 binds phosphatidylserine and phosphatidylethanolamine [8]. The CH domain of EHBP1L1 interacts with several F-actin-binding proteins [14]. This interaction of EHBP1L1 and F-actin suggests a function that connects the process of intracellular transport through binding with the actin cytoskeleton through its CH domain [18], which is necessary for intracellular transport in cells. In this study, we have shown that the six TPR domains of KLC1 interacted with the coiled-coil domain of EHBP1L1.

What would the interaction between KLC1 and EHBP1L1 mean? We are able to suggest one possibility is that

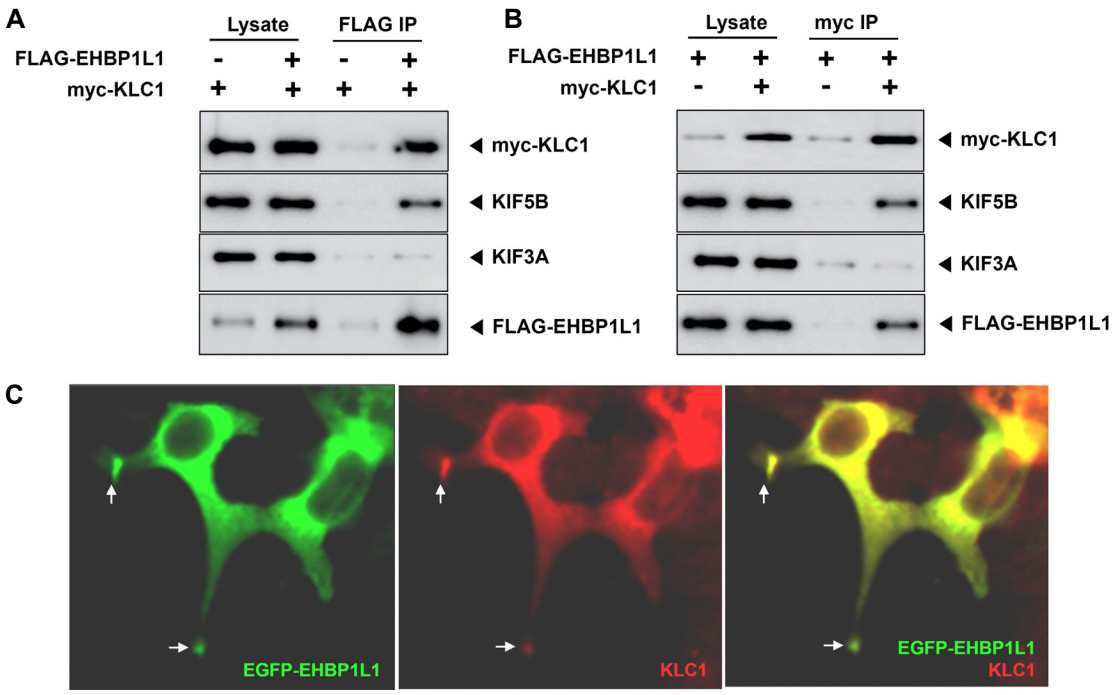

Fig. 3. Co-immunoprecipitation and co-localization of KLC1 and EHBP1L1 in mammalian cells. (A and B) HEK-293T cells were transiently transfected with FLAG- EHBP1L1 and myc-KLC1 plasmids as indicated. Cell lysates were immunoprecipitated with (A) monoclonal anti-FLAG antibody or (B) anti-myc antibody. Precipitates were immunoblotted with anti-KLC1, KIF5B, KIF3A and FLAG antibodies. EHBP1L1 specifically co-precipitated KLC1 and KIF5B, but not with KIF3A. (C) HEK-293T cells were transiently transfected with EGFP-EHBP1L1 and KLC1 plasmids. Twenty-four hours after transfection, cells were subjected to immunofluorescence with anti-KLC1 antibody. KLC1 and EHBP1L1 were observed in the same subcellular region in cells. KLC, kinesin light chain; EHBP1L1, EH domain-binding protein 1-like 1; KIF, kinesin superfamily proteins; EGFP, enhanced green fluorescent protein; IP, immunoprecipitation.

EHBP1L1 may be an adaptor protein that links kinesin 1 and endosomal vesicles. EHBP1L1 links the Bin1-dynamin complex, which generates membrane curvature and excise the vesicle from the endocytic recycling compartment [11]. EHBP1L1 localizes to the endocytic recycling compartment and involves in apical transport with Rab8-EHBP1L1-Bin1 complex in epithelial cells as well as HeLa cells [2, 11]. Another possibility is that EHBP1L1 may be an adaptor protein that links kinesin 1 and Rab8-positive lysosomes/endosomes. EHBP1L1 was first identified as a Rab8 binding protein [11]. Rab8 plays a role in exocytosis toward the polarized plasma membrane in eukaryotic cells [2, 11]. Rab8s knockout intestine cell show accumulated cargo proteins in lysosome, which suggests that Rab8 is involved in the lysosomal transport [11]. EHBP1L1 binds to Rab8s and Bin1 [11]. This EHBP1L1-interacting protein complex, Rab8s-EHBP1L1-Bin1 localizes to the Rab8-positive lysosomes/endosomes [2, 11]. Although we did not determine the specific character of the Rab8-positive lysosomes/endosomes as kinesin 1-cargo, the available data suggest that EHBP1L1 play a role as an adaptor protein for Rab8-positive lysosomes/endosomes by kinesin 1.

In many cases, various different types of cargos such as mitochondria, and small vesicles interact with adaptor proteins/scaffolding proteins that mediate the attachment to kinesin 1 [4, 5, 17]. Thus, EHBP1L1 may serve as a scaffolding protein that links kinesin 1 and Rab8-positive lysosomes/endosomes or endosomal vesicles. Taken together, we suggest that the kinesin 1-EHBP1L1 interaction may play crucial role(s) in intracellular transport of Rab8-positive lysosomes/endosomes or endosomal vesicles.

Acknowledgment

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The Conflict of Interest Statement

The authors declare that they have no conflicts of interest with the contents of this article.

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초록 : Kinesin Light Chain 1 (KLC1)의 Tetratricopeptide Repeat (TPR) 도메인과 Rab effector, EHBP1L1의 결합

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Kinesin 1은 미세소관을 따라 plus말단으로 이동하는 모터단백질로 세포내 물질 수송에 관여한다. Kinesin 1은 경쇄단위체(light chain subunit)를 통하여 운반체들인, 세포내 소기관, 다양한 소포체, 신경전달물질 수용체 단백질, 세포신호전달 단백질과 여러 단백질 복합체들과 결합하여 운반하는 kinesin superfamily protein (KIFs)의 한 종류이다. Kinesin light chains 1 (KLC1)은 모터 기능이 없는 단위체로서 kinesin heavy chain (KHC)과 결합한다. KLC1은 다양한 매개단백질들과 결합하지만 아직 결합하는 매개단백질이 충분히 밝혀지지 않았다. 본 연구에서는 KLC1의 tetratricopeptide repeat (TPR) 영역과 결합하는 단백질을 분리하기 위하여 효모 two-hybrid 탐색한 결과 EH domain-binding protein 1-like 1 (EHBP1L1) 을 분리하였다. EHBP1L1은 KLC1의 TPR 영역을 포함한 부위와 결합하지만 KIF5B (kinesin 1의 모터 단백질)과 KIF3A (kinesin 2의 모터 단백질)와는 결합하지 않았다. 또한 KLC1은 EHBP1L1의 C-말단에 존재하는 coiled-coil 도메인과 결합하였으며, 다른 EHBP1L1의 isoform인 EHBP1과는 결합하지 않았다. KLC1은 GST와는 결합하지 않지만 GST-EHBP1L1과 GST-EHBP1L1-coiled-coil domain과는 결합하였다. HEK-293T세포에 EHBP1L1과 KLC1을 동시에 발현시켰을 때 두 단백질은 세포 내에서 같은 부위에 존재하며, EHBP1L1을 면역침강한 결과 KLC1뿐만 아니라 KIF5B와도 같이 침강함을 확인하였다. 이러한 결과들은 kinesin 1은 EHBP1L1이 결합한 운반체를 수송함을 시사한다.